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## (57) Abstract

Chimaeric pseudovirus particles and a method for producing a foreign protein using the same are disclosed. The pseudovirus particles comprise a protein (e.g. a coat protein) having a viral portion and a non-viral portion, and a nucleic acid (optionally chimaeric) to stabilize the aggregation of the protein, and create a helical ribonucleocapsid with the structure and symmetry approaching the native virus.

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1     Virus-like particle.

2     This invention relates to a virus-like particle,  
3     especially to a pseudovirus particle, and to a method  
4     for the production of a chimaeric protein using such  
5     virus-like particles. The protein can be a capsid  
6     protein which can self assemble *in vivo* with the  
7     nucleic acid (which may be chimaeric) to form the  
8     virus-like particles.

9  
10    Pseudovirus particles are virus-like particles  
11    comprising viral coat protein subunits and a portion of  
12    the wild-type viral nucleic acid. Pseudoviruses may  
13    also include foreign nucleic acid. The coat protein  
14    can be wild-type, modified or chimaeric. A pseudovirus  
15    may lack at least a portion of the wild-type viral  
16    nucleic acid (or may possess a non-functional analogue  
17    of the wild-type nucleic acid) and this commonly  
18    renders the pseudovirus incapable of some function  
19    which is characteristic of the wild-type virus, such as  
20    replication. Alternatively or additionally, other  
21    genes may be missing or disabled, and the pseudovirus  
22    may be, for example, replication competent but  
23    incapable of cell-cell movement. The missing or  
24    dysfunctional gene(s) can be provided on the genome of  
25    a host cell or on a plasmid etc present in the host  
26    cell, thereby restoring the function of the wild-type  
27    virus to the pseudovirus when in the host cell.

28  
29    The physical properties of the pseudovirus particle  
30    such as shape, symmetry, nucleic acid:protein ratio are  
31    usually similar to or identical with the wild-type

1 virus from which the pseudovirus is derived, although  
2 particle length and width can be influenced by nucleic  
3 acid length and coat protein composition respectively.

4  
5 According to the present invention there is provided a  
6 virus-like particle comprising nucleic acid and  
7 protein, the protein having a first (viral) portion and  
8 a second (non-viral) portion.

9  
10 The term "virus-like particle" refers to self-  
11 assembling particles which have a similar physical  
12 appearance to virus particles and includes  
13 pseudoviruses. Virus-like particles may lack or  
14 possess dysfunctional copies of certain genes of the  
15 wild-type virus, and this may result in the virus-like-  
16 particle being incapable of some function which is  
17 characteristic of the wild-type virus, such as  
18 replication and/or cell-cell movement.

19  
20 The nucleic acid can be DNA or RNA, according to the  
21 genome of the virus from which the virus-like particle  
22 is derived. The nucleic acid may comprise an origin-  
23 of-assembly sequence (OAS) by which we mean a nucleic  
24 acid sequence which permits initiation of assembly of  
25 the protein and nucleic acid into virus-like particles.

26  
27 Further according to the invention there is provided a  
28 method of producing a protein having a first (viral)  
29 portion and a second (non-viral) portion, the method  
30 comprising expressing the protein in a cell, providing  
31 a nucleic acid sequence capable of assembly with the  
32 protein into a virus-like particle (VLP), and  
33 permitting *in vivo* assembly of the protein and nucleic  
34 acid into VLPs.

35  
36 The virus-like particles can be purified from the cell

1 by standard techniques such as centrifugation etc, and  
2 the chimaeric protein can optionally be cleaved to  
3 release the second portion from the first portion, or  
4 separated entirely from the nucleic acid. If the  
5 chimaeric protein is left attached to the virus-like  
6 particle, the whole virus-like particle can also be  
7 used for presentation of peptide epitopes for  
8 vaccination of animals, the production of therapeutic  
9 or industrial proteins and polypeptides and/or the  
10 delivery of therapeutic nucleic acid molecules  
11 (optionally targeted delivery), such as ss or ds DNA or  
12 RNA, including antisense molecules.

13  
14 The nucleic acid can advantageously be provided from a  
15 plasmid in the cell, possibly by transcription of such  
16 a plasmid. The protein may be encoded by the same or  
17 another plasmid in the cell. Alternatively, one or  
18 both of the nucleic acid and protein can be coded from  
19 the genome of the cell.

20  
21 The cell is preferably a bacterium such as *E. coli*  
22 although other forms of bacteria and other cells may be  
23 useful, such as mammalian cells, plant cells, yeast  
24 cells and insect cells. The cell may be a natural host  
25 cell for the virus from which the virus-like particle  
26 is derived, but this is not necessary.

27  
28 The use of a cell for the assembly of the virus-like  
29 particle *in vivo* enables facile cell handling  
30 techniques to be employed to facilitate purification of  
31 virus-like particles and purification of protein. In  
32 addition, where it is desired to produce a second  
33 portion protein which is toxic to some cells (eg plant  
34 cells) a different (eg bacterial) cell may be employed.

35  
36 The nucleic acid is preferably chosen in accordance

1 with its ability to assemble with the viral protein.  
2 For example, the virus-like particle may be derived  
3 from tobacco mosaic virus (TMV). In such a case, the  
4 first portion of the protein is preferably derived from  
5 TMV coat protein (CP), and the nucleic acid has at  
6 least an OAS of eg 75 or more nucleotides derived from  
7 TMV RNA. The sequence of the remainder of the nucleic  
8 acid is not important, and it can be chosen to code for  
9 the chimaeric protein or may be of some other eg  
10 unrelated or therapeutic sequence. The inclusion of  
11 nucleic acid in the virus-like particle means that the  
12 particle is of helical symmetry and more stable than  
13 simple aggregations of protein (eg planar, stacked or  
14 helical arrays), which are normally created at low pH  
15 *in vitro* from purified TMV coat protein, and can  
16 dissociate outside a narrow pH range. Also, the length  
17 of the particle can be selected by specifying a  
18 particular length of nucleic acid. This results in a  
19 more uniform range of particle sizes, which has  
20 advantages in purification procedures such as  
21 centrifugation, and in defining and regulating the  
22 quality control for products for medical use.

23  
24 A further advantage with the use of nucleic acid in the  
25 assembly of virus-like particles is that the resultant  
26 particle can have a regular multivalent and true  
27 helical structure which can be more immunogenic than an  
28 aggregation of protein or free subunits of protein.  
29 The greater stability of the particle can also provide  
30 longer access to the immune system in certain  
31 embodiments.

32  
33 The second portion of the chimaeric protein is  
34 preferably disposed on the outer surface of the virus-  
35 like particle. Thus where the particle is derived from  
36 TMV, the second portion can be disposed on the amino or

1 carboxy terminus, or inserted in eg an internal loop  
2 disposed on the outer surface of the CP. This can  
3 result in improved assembly as compared with the  
4 assembly of particles having a second portion on  
5 another location of the CP, and can enhance immune  
6 recognition of the second portion on the particle  
7 surface, which is useful for embodiments where the CP  
8 is an immunogen such as a vaccine. In certain cases it  
9 may be possible to provide large second portion  
10 proteins.

11

12 It is advantageous to use a virus which is flexuous (ie  
13 which can bend easily) since chimaeric proteins with  
14 large second portions may be able to assemble more  
15 easily into virus particles which are flexuous than  
16 those which are rigid. PVX is preferred since it forms  
17 a flexuous particle.

18

19 A linker peptide can be incorporated between the first  
20 and second portions and may have the function of  
21 spacing the two portions from one another, reducing  
22 steric restrictions. Optionally the linker peptide may  
23 contain a cleavage site.

24

25 The term "cleavage site" refers to a short sequence of  
26 amino acids which is recognisable and subsequently  
27 cleavable by eg a proteolytic enzyme or by chemical  
28 means. Suitable proteolytic enzymes include trypsin,  
29 pepsin, elastase, factor Xa etc. Alternatively the  
30 cleavage site may be vulnerable to cleavage by other  
31 means, for example by addition of chemicals such as  
32 cyanogen bromide (CNBr) or acids.

33

34 The term "cleavage site" may also include sequences  
35 that self-leave such as the FMDV (Foot and Mouth  
36 Disease Virus) 2A protease.

1 The cleavage site may be an integral part of either the  
2 first or second portion. Hence either/or both of the  
3 portions may include an integral cleavage site.

4  
5 The second portion protein may be a short oligopeptide  
6 (10-40 amino acids) or a relatively large polypeptide  
7 eg over 10kDa. Proteins of 25-30 kDa may also be  
8 suitable for production by the method of the invention.

9  
10 The first (viral) portion of the chimaeric protein may  
11 be any protein, polypeptide or parts thereof, derived  
12 from a viral source including any genetically modified  
13 versions thereof (such as deletions, insertions, amino  
14 acid replacements and the like). In certain  
15 embodiments the first portion will be derived from a  
16 viral coat protein (or a genetically modified version  
17 thereof). Mention may be made of the coat protein of  
18 Potato Virus X as being suitable for this purpose.  
19 Preferably the first portion has the ability to  
20 assemble into virus-like particles by first-  
21 portion/first portion association. Thus, a chimaeric  
22 protein molecule can assemble with other chimaeric  
23 protein molecules or with wild-type coat protein into a  
24 chimaeric virion.

25  
26 In a preferred embodiment of the invention the particle  
27 is derived from a tobamovirus such as tobacco mild  
28 green mosaic virus TMGMV), tobacco mosaic virus (TMV),  
29 or from a potexvirus such as PVX, and in such an  
30 embodiment, the second portion is preferably disposed  
31 at or adjacent the N-terminus of the coat protein. In  
32 PVX, the N-terminus of the coat protein is believed to  
33 form a domain on the outside of the virion.

34  
35 The second portion of the chimaeric protein may be any  
36 protein, polypeptide or parts thereof, including any



1 genetically modified versions thereof (such as  
2 deletions, insertions, amino acid replacements and the  
3 like) derived from a source other than the virus from  
4 which the first portion is derived. In certain  
5 embodiments the second portion or the protein derived  
6 therefrom is a biologically active or otherwise useful  
7 molecule. The second portion or the protein derived  
8 therefrom may also be a diagnostic reagent, an  
9 antibiotic or a therapeutic or pharmaceutically active  
10 agent. Alternatively the second portion protein may be  
11 a food supplement.

12  
13 It is not necessary for the first portion to comprise a  
14 whole virus coat protein, but this remains an option.  
15 Some non-essential amino acids could be removed during  
16 construction of the CP gene.

17  
18 The virus particle may be formed by the assembly of  
19 chimaeric proteins only or by the mixed assembly of  
20 chimaeric proteins together with some unmodified or  
21 less modified forms of the naturally occurring wild-  
22 type coat protein which forms the basis of the first  
23 portion. For a mixed virus particle of the latter  
24 type, there must be present polynucleotide(s) encoding  
25 the chimaeric protein and the naturally occurring coat  
26 protein. The appropriate protein-coding sequence(s)  
27 may be arranged in tandem on the same molecule, or  
28 could be generated by differential RNA splicing  
29 Alternatively, the different proteins could be  
30 translated from the same nucleotide sequence and  
31 modified later, eg by *in vivo* processing such as self  
32 cleavage. An example of this is the provision of a  
33 chimeric CP gene encoding eg GFP-2A-CP fusion protein,  
34 which is expressed from a single gene (eg on a plasmid,  
35 from the genome of the cell, or from the RNA of the  
36 VLP) and which self cleaves a variable number of the

1 translated proteins into separate GFP and CP, a  
2 proportion of the translated proteins remaining  
3 uncleaved as GFP-2A-CP. Thus a heterologous mixture of  
4 CPs can be assembled into a VLP, with eg every 10th CP  
5 bearing a second portion, and the remaining CPs being  
6 cleaved, native (or substantially native) CPs. Thus  
7 the potential problems with stearic hindrance which  
8 might occur if all the CPs were chimaeric can be  
9 overcome. Suitable co-translational cleavage sequences  
10 can be chosen for particular cell types. The  
11 efficiency of the co-translational cleavage can be  
12 modified to produce the required proportion of  
13 cleaved/whole CPs in the assembled VLP.

14  
15 An advantage is gained by using a virus which forms a  
16 helical particle with a relatively large pitch. PVX  
17 has a pitch of 3.4nm and is to be preferred over  
18 viruses with a lower pitch. Virus particles with  
19 higher pitches may be able to accommodate larger  
20 protein insertions on their surfaces since their coat  
21 proteins assemble with more space between them than  
22 coat proteins of viruses with lower pitches.

23  
24 The method can be used for expression of metabolic  
25 enzymes for pathway engineering, nutritional  
26 supplements (eg hi-met proteins), anti-potato cyst  
27 nematode lectins, gut protease inhibitors, anti-  
28 botrytis agents, PGIPs, anti-insect *Bacillus*  
29 *thuringiensis* toxin and herbicide resistance agents,  
30 industrial enzymes, pharmaceuticals, therapeutic  
31 proteins and nucleic acids, and as bioreactors.

32  
33 While modifications and improvements may be  
34 incorporated without departing from the scope of the  
35 invention, embodiments will now be described by way of  
36 the following examples and with reference to the

1 accompanying drawings in which:

2

3 Fig 1 is a schematic representation of the plasmid  
4 pA27;

5 Fig 2 is an SDS PAGE analysis of proteins from purified  
6 TMV and pseudovirions. Samples were electrophoresed on  
7 an SDS/PAGE gel and silver stained. Lane 1, purified  
8 TMV. Lane 2, VLPs purified from E. coli BL21(DE3)  
9 cells transformed with plasmids pA27 and pLys102. The  
10 positions of coelectrophoresed marker proteins and  
11 their molecular weights in kDa are shown to the left;  
12 Fig 3 is an electron microscope image of VLPs. VLPs  
13 purified from E. coli BL21(DE3) cells transformed with  
14 plasmids pA27 and pLys102 were negatively stained with  
15 2% sodium phosphotungstate pH 5.0 and viewed in the  
16 electron microscope. Magnification x 20,000;

17 Fig 4 shows sequence information for LITMUS 39 plasmids  
18 used in Example 2;

19 Fig 5 shows a schematic representation of cDNA  
20 constructs used in Example 2;

21 Fig 6 shows immunoblot analysis of extracts of leaves  
22 probed with anti-CP antiserum; and

23 Fig 7 shows immunoblot analysis of virus prepared from  
24 plants infected with a VLP.

25

26 Example 1:

27 A sequence encoding two glycine residues and an eight  
28 amino acid antigenic epitope (EQPTTRAQ) from VP1 of  
29 poliovirus type 3 [1] was fused to the 3' end of a  
30 synthetic gene coding for the tobacco mosaic virus  
31 (TMV) coat protein by PCR amplification with mutagenic  
32 primers. The plasmid pTMVCP [1] was used as a template  
33 for amplification with primers P1311 (5' AAG-AAT-TCA-  
34 TAT-GTC-TTA-TTC-GAT-TAC-C 3') and P1312 (5' AAG-GAT-  
35 CCT-CAC-TGA-GCA-CGA-GTA-GTC-GGC-TGT-TCA-CCA-CCA-GTT-  
36 GCC-GGG-CCC-GAG 3'). The amplification product was

1 treated with T4 DNA polymerase to make it blunt-ended  
2 and ligated into *EcoRV* digested pKR [2]. The ligation  
3 products were transformed into *E. coli* strain JM101.  
4 Transformants were screened for the desired plasmid,  
5 pA11, containing the gene encoding the modified TMV  
6 coat protein.

7  
8 To enable expression of the modified TMV coat protein  
9 in *E. coli* a fragment encompassing the modified gene  
10 was cloned into an expression vector, under the  
11 transcriptional control of T7 promoter and T<sub>0</sub>  
12 terminator sequences. The plasmid pA11 was digested  
13 with *NdeI* and *BamHI* and the 510 base pair fragment  
14 released was cloned between the same sites of pET3a  
15 [3]. The nucleotide sequence of the resulting plasmid,  
16 pA27 (Figure 1), in the region encoding the eight amino  
17 acid epitope and the linker of two glycine residues,  
18 was confirmed by nucleotide sequence determination.

19  
20 In Figure 1, sequence encoding TMV coat protein and ten  
21 amino acid peptide fused to the carboxy-terminus are  
22 indicated by boxes marked TMV CP and PEP respectively.  
23 Restriction endonuclease sites used for the  
24 introduction of the modified TMV coat protein gene into  
25 the plasmid pET3a are indicated above. The T7 promoter  
26 and T<sub>0</sub> terminator sequences from the plasmid pET3a are  
27 indicated by a double thickness arrow and line  
28 respectively. The nucleotide sequence of the 3' end of  
29 the modified TMV coat protein gene and the amino acids  
30 encoded by this sequence are shown below. The  
31 nucleotide sequence encoding the additional ten amino  
32 acids and the amino acids themselves are shown in bold.

33  
34 To obtain expression of the modified TMV coat protein  
35 and production of pseudovirions the plasmid pA27 was  
36 transformed into *E. coli* BL21(DE3) cells that had

1 previously been transformed with the plasmid pLys102  
2 [4]. The plasmid pLys102 produces a chimaeric RNA  
3 transcript encoding chloramphenicol acetyl transferase  
4 and containing the TMV origin-of-assembly sequence,  
5 which when co-synthesized with TMV coat protein in *E.*  
6 *coli* directs the assembly of pseudovirus particles of  
7 70nm length (modal) and 18nm diameter. That plasmid  
8 pA27 directed the synthesis of modified TMV coat  
9 protein was confirmed by SDS/PAGE analysis of IPTG  
10 induced bacterial lysates [4]. Production of a TMV coat  
11 protein-related protein with a slightly lower mobility  
12 than unmodified TMV coat protein was detected by  
13 Coomassie blue staining and immunoblotting of SDS/PAGE  
14 gels as described by Hwang et al. [4].  
15  
16 VLPs containing the modified TMV coat protein were  
17 purified using a protocol based on that described by  
18 Hwang et al. [4]. Colonies of BL21(DE3) co-transformed  
19 with pA27 and pLys102 were used to inoculate 5 ml of  
20 M9ZB medium supplemented with 100 µg/ml ampicillin and  
21 35 µg/ml chloramphenicol. Cultures were grown overnight  
22 at 37°C. The bacteria were pelleted from the overnight  
23 cultures and used to inoculate 500 ml of M9ZB medium  
24 supplemented with ampicillin and chloramphenicol. The  
25 large-scale cultures were grown at 37°C until mid-log  
26 phase ( $A_{600} = 0.7$ ). Cultures were induced with 0.4 mM  
27 IPTG and incubated at 30°C for eighteen hours. Cells  
28 were harvested by centrifugation (4800 x g, 4°C, 6  
29 min). Bacterial pellets were resuspended in 3ml of TE  
30 (10 mM Tris-HCl pH 7.5 / 1 mM EDTA) and incubated with  
31 lysozyme (0.4 mg/ml) at 20°C for 60 min. Bacteria were  
32 lysed by addition of 4 ml 40% w/v sucrose in TE and  
33 then 16 ml of TE. DNase I was added to 6.5 µg/ml and  
34 the lysates incubated at 37°C for 90 min. Bacterial  
35 debris was removed by centrifugation (20800 x g, 4°C,  
36 30 min). The resulting supernatants were extracted with

1 10 ml of chloroform and the two phases separated by  
2 centrifugation (9200 x g, 4°C, 10 min). 3.7 ml of 5M  
3 NaCl and 2.63 ml of 40% polyethylene glycol (average  
4 molecular weight 6000) were added to 20 ml of the  
5 aqueous phase. The solutions were mixed and incubated  
6 on ice for 60 min. Precipitated material was collected  
7 by centrifugation (20800 x g, 4°C, 15 min). The  
8 pelleted material was resuspended in 1 ml of TE.  
9 Insoluble material was removed by centrifugation (16000  
10 x g, 4°C, 5 min). The supernatant was centrifuged  
11 (160000 x g, 4°C, 120 min) on a sucrose gradient (10-  
12 40% w/v in TE). Fractions were collected from the  
13 gradients and those containing helical TMV-like  
14 particles, assessed by double-antibody sandwich ELISA  
15 with a mouse monoclonal antibody specific for an  
16 epitope in the TMV coat protein helix as described by  
17 Hwang et al. [4], were pooled for further purification.

18  
19 VLPs were collected by centrifugation (235,000 x g,  
20 15°C, 150 min). Pelleted pseudovirions were resuspended  
21 in 0.5 ml of TE. Insoluble material was removed by  
22 centrifugation (840 x g, 4°C, 5 min). The supernatant  
23 was centrifuged (189,000 x g, 15°C, 120 min) on a CsCl  
24 gradient (10-40% (wt/wt) in TE). Bands containing  
25 pseudovirus were collected from the gradients and  
26 dialyzed against 50 mM sodium phosphate pH 7.0.

27  
28 The yield of VLPs was estimated by measuring the  
29 absorption at 260 nm. The final yield of pseudovirus  
30 was 5.8 mg from 500 ml of culture. The purity of the  
31 pseudovirus preps was assessed by silver staining of  
32 samples electrophoresed on SDS/PAGE gels (Figure 2). On  
33 SDS/PAGE gels the unmodified TMV coat protein produced  
34 by pET302 and the modified coat protein produced by  
35 pA27 migrate relative to protein standards (Bio-Rad)  
36 with apparent molecular weights of 20.9 kDa and 22.6

1 kDa respectively. The predicted molecular weights for  
2 these two proteins are 17.7 kDa and 18.6 kDa  
3 respectively.

4  
5 The integrity of the pseudovirus preparations was  
6 assessed by negative staining of pseudovirus samples  
7 with 2% sodium phosphotungstate and observation of the  
8 stained samples in the electron microscope (Figure 3).  
9 Pseudovirus preparations were diluted to 1 mg / ml in  
10 50 mM sodium phosphate pH 7.0 for immunization of mice.  
11

12 Example 2:

13 A plasmid containing the tobacco mild green mosaic  
14 virus (TMGMV) coat protein (CP) gene and 3'  
15 untranslated region (UTR) was produced to facilitate  
16 the production of green fluorescent protein (GFP),  
17 foot-and-mouth disease virus 2A, TMGMV CP gene fusions.  
18 A 955 base pair (bp) fragment containing the TMGMV CP  
19 and 3' UTR was PCR amplified from the plasmid 30B (W.O.  
20 Dawson, Citrus Research and Education Center) using the  
21 primers TMGMV-CP-Apa (5' CAA-TGG-GCC-CTA-TAC-AAT-CAA-  
22 CTC-T 3') and M13-Reverse (5' AGC-GGA-TAA-CAA-TTT-CAC-  
23 ACA-GGA 3'). The primer TMGMV-CP-Apa was designed to  
24 mutagenize the sequence coding for the initiating  
25 methionine and first proline codon of the TMGMV CP to  
26 an ApaI restriction enzyme site. This results in the  
27 conversion of the methionine codon to a glycine codon,  
28 but maintains the proline codon. The 837bp fragment  
29 released by digestion of the PCR amplification product  
30 with the restriction endonucleases ApaI and KpnI was  
31 cloned into the 3322bp fragment released by digestion  
32 of pSL1180 (Pharmacia) digested with the same  
33 restriction endonucleases and treated with calf  
34 intestinal alkaline phosphatase. The resulting plasmid  
35 was named pSL.TMGMV-CP-UTR.

36

1 CFP-2A-TMG MV CP gene fusions were produced by cloning  
2 DNA fragments containing GFP-2A fusions into pSL.TMG MV-  
3 CP-UTR adjacent to the codon for the first proline in  
4 the TMG MV CP gene. A selection of LITMUS 39 (New  
5 England Biolabs) based plasmids containing GFP-2A-  
6 potato virus X CP gene fusions were used as sources for  
7 the GFP-2A gene fusion.

8  
9 The nucleotide sequence and amino acids encoded by the  
10 different LITMUS 39 based plasmids between the carboxy-  
11 terminal lysine codon of the GFP gene and the amino-  
12 terminal proline codon of the PVX CP gene are shown in  
13 Figure 4.

14  
15 These plasmids contain a variety of sequences coding  
16 for different 2A amino acid sequences between the  
17 carboxy-terminal lysine codon of GFP and the first  
18 proline codon of the PVX CP. Fragments of between 900  
19 and 1050bp were PCR amplified from the plasmids pLit,  
20 GFP-2A<sub>16H</sub>-CP, pLit.GFP-2A<sub>16K</sub>-CP, pLit.GFP-2A<sub>23H</sub>-CP and  
21 pLit.GFP-2A<sub>38K</sub>-CP using the primers GFP-5'-Sal (5' TCA-  
22 ATC-GTC-GAC-ATG-AGT-AAA-GGA-GAA-GAA 3') and N3#4 (5'  
23 TGT-ACT-AAA-GAA-ATC-CCC-ATC-C 3'). The primer GFP-5'-  
24 Sal introduces a Sall restriction enzyme site upstream  
25 of the initiating methionine codon of the GFP gene.  
26 Fragments containing the GFP gene fused to the  
27 different 2A sequences were released by digestion of  
28 the PCR amplification products with Sall and ApaI and  
29 ligated into the large fragment released by digestion  
30 of pSL.TMG MV-CP-UTR with the same restriction enzymes  
31 and treated with calf intestinal phosphatase. The  
32 resulting plasmids were digested with Sall and BstEII  
33 and the released fragments containing the GFP-2A-TMG MV  
34 CP gene fusion and TMG MV UTR were introduced into the  
35 plasmid 30B digested with XhoI and BstEII to regenerate  
36 full-length TMV based clones. Thus the final clones



1 comprise wild-type TMV strain U1 sequence up to  
2 position 5757 in the CP gene, with the exception of a  
3 mutagenized CP initiating methionine codon, followed by  
4 a short polylinker sequence, the GFP-2A-TMGMV CP gene  
5 fusions and the TMGMV 3' UTR.

6  
7 Figure 5 shows a schematic representation of viral cDNA  
8 constructs used in this example. Boxes represent  
9 coding sequences. The genes for the three viral  
10 proteins common to all constructs are indicated by  
11 their predicted Mr values (K=kDa). The genes for the  
12 green fluorescent protein, 2A oligopeptide and TMGMV CP  
13 are indicated by GFP, 2A and CP respectively.  
14 Restriction enzyme sites used in the cloning procedures  
15 are indicated above.

16  
17 *In vitro* run-off transcripts were synthesized from KpnI  
18 linearized plasmids p30B.GFP-2A<sub>168</sub>-CP, p30B.GFP-2A<sub>16K</sub>-CP,  
19 p30B.GFP-2A<sub>238</sub>-CP, p30B.GFP-2A<sub>58K</sub>-CP and p30B.GFP, a  
20 derivative of p30B that has had the GFP gene introduced  
21 into the unique XhoI site of P30B, which expresses free  
22 GFP. The transcripts derived from all the plasmids  
23 were infectious when inoculated onto *Nicotiana*  
24 *benthamiana* plants; virus derived from transcript-  
25 infected plants is referred to subsequently by the name  
26 of the progenitor plasmid without the "p" prefix.  
27 Following inoculation, all the viruses caused the  
28 development of fluorescent regions which were first  
29 detectable by eye under UV illumination between three  
30 and four days post inoculation. Subsequent long  
31 distance movement of the virus led to the appearance of  
32 green fluorescence in systemically infected leaves.  
33 The appearance of fluorescence in systemically infected  
34 leaves occurred at a similar time, nine days post  
35 inoculation, for plants infected with 30B.GFP, 30B.GFP-  
36 2A<sub>168</sub>-CP and 30B.GFP-2A<sub>16K</sub>-CP, but was delayed for

1 30B.GFP-2A<sub>23H</sub>-CP and 30B.GFP-2A<sub>58K</sub>-CP.

2

3 Western blotting of protein extracts from systemically  
4 infected *N. benthamiana* leaves, probed with rabbit  
5 polyclonal antisera raised against TMV CP (Figure 6),  
6 detected two protein species in each of the 30B.GFP-2A-  
7 CP infected samples. This result indicated that the  
8 modified viruses were producing a GFP-2A-CP fusion  
9 protein, the *in vivo* processing of which resulted in  
10 the production of a GFP-2A fusion protein and free  
11 TMGMV CP. For 30B.GFP-2A<sub>16H</sub>-CP, 30B.GFP-2A<sub>16K</sub>-CP and  
12 30B.GFP-2A<sub>58K</sub>-CP the majority of CP related protein  
13 produced was in the unfused form. Protein was prepared  
14 from mock-inoculated control plants (lane 1) or from  
15 plants inoculated with *in vitro* transcripts synthesized  
16 from plasmid DNAs (p30B.GFP, lane 2; p30B.GFP-2A<sub>23H</sub>-CP,  
17 lane 3; p30B.GFP-2A<sub>16H</sub>-CP, lane 4; p30B.GFP-2A<sub>16K</sub>-CP, lane  
18 5; p30B.GFP-2A<sub>58K</sub>-CP, Lane 6). Lane 7 contains 125ng of  
19 TMGMV CP. The predicted Mr values of TMGMV CP, GFP and  
20 GFP-2A-CPs are 17.5 kDa, 26.9 kDa and between 46 and 52  
21 kDa, respectively. The Mr values of standards (X10<sub>3</sub>)  
22 are shown on the left.

23

24 The observation that the modified viral constructs were  
25 capable of rapid systemic movement like 30B.GFP  
26 suggested that they were also capable of virus particle  
27 formation. To confirm that this was the case  
28 homogenates were prepared by grinding fluorescent  
29 inoculated leaf tissue from plants infected with  
30 30B.GFP and 30B.GFP-2A<sub>23H</sub>-CP in a "mini-mortar" with  
31 50mM phosphate buffer pH 6.5. The homogenates were  
32 applied to a carbon coated grid and stained with 2%  
33 sodium phosphotungstate pH 6.5 prior to observation in  
34 the electron microscope. 30B.GFP-2A<sub>23H</sub>-CP was found to  
35 produce rod-shaped particles like those produced by  
36 30B.GFP. To test whether the particles produced by

1 30B.GFP-2A<sub>238</sub>-CP had incorporated GFP-2A-CP fusion  
 2 protein as well as free TMGMV CP immunotrapping  
 3 (Roberts 1986, in Electron microscopy of proteins,  
 4 Academic Press) was performed with rabbit polyclonal  
 5 antisera raised against GFP and TMV.CP. While 30B.GFP  
 6 infected tissue showed enhanced trapping with the TMV-  
 7 CP antisera, but not with the GFP antisera, 30B.GFP-  
 8 2A<sub>238</sub>-CP infected tissue showed enhanced trapping with  
 9 both antisera (Table 1). This result suggested that  
 10 the modified virus was capable of incorporating GFP-2A-  
 11 CP fusion protein into particles.

12  
 13 Table 1  
 14 Number of particles/1000 $\mu$ m<sup>2</sup>

16 Coating		
	17 30B.GFP	30B.GFP-2A <sub>238</sub> -CP
18		
19 None	223 +/- 57.0	3.5 +/- 1.33
20		
21 TMV CP	4690 +/- 1200	58.0 +/- 3.16
22		
23 GFP	112 +/- 9.45	67.5 +/- 15.2
24		

25 To confirm this a virion extraction (Kearney et al, in  
 26 Plant Molecular Biology Manual L1:1-16, Kluwer Academic  
 27 Publishers) was performed on fluorescent, systemically  
 28 infected tissue of plants infected with 30B.GFP-2A<sub>168</sub>-  
 29 CP. Western blot analysis (Fig 7) of the virus  
 30 preparation with GFP (B) and TMV CP (A) antisera  
 31 demonstrated that the virus contained TMGMV CP and CGP-  
 32 2A-CP fusion protein but no GFP-2A fusion protein. Mr  
 33 values shown on left of Fig 7 ( $\times 10^{-3}$ ) Thus the GFP-2A-  
 34 CP fusion protein was assembled with free TMGMV CP into  
 35 virus particles.

36

- 1 Modifications and improvements can be incorporated
- 2 without departing from the scope of the invention.
- 3

1 Documents incorporated herein by reference:

2

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8 [2] Waye, M.M.Y., Verhoeyen, M.E., Jones, P.T. and  
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16 U.S.A., 91, 9067-9071. WO 94/10329 (Rutgers  
17 University), see particularly deposit information  
18 therein.

## 1     Claims:

2

3     1     A method of producing a protein having a first  
4     (viral) portion and a second (non-viral) portion, the  
5     method comprising expressing the protein in a cell,  
6     providing a nucleic acid sequence capable of assembly  
7     with the protein into a virus-like particle (VLP), and  
8     permitting *in vivo* assembly of the protein and nucleic  
9     acid into VLPs.

10

11     2     A method as claimed in claim 1, wherein the VLPs  
12     are subsequently purified from the cell.

13

14     3     A method as claimed in claim 1 or claim 2, wherein  
15     after assembly the protein is cleaved to release the  
16     second portion from the first portion, or is separated  
17     entirely from the nucleic acid.

18

19     4     A method as claimed in any preceding claim,  
20     wherein the nucleic acid is provided from a plasmid.

21

22     5     A method as claimed in claim 4, wherein the  
23     protein is encoded by the same or another plasmid in  
24     the cell, or from the genome of the cell.

25

26     6     A method as claimed in any preceding claim,  
27     wherein the cell is selected from bacterial cells,  
28     mammalian cells, plant cells, yeast cells and insect  
29     cells.

30

31     7     A method as claimed in claim 6, wherein the cell  
32     is a natural host cell for the virus from which the  
33     virus-like particle is derived.

34

35     8     A method as claimed in any preceding claim,  
36     wherein the second portion of the protein is disposed

1 on the outer surface of the VLP.

2

3 9 A method as claimed in any preceding claim,  
4 wherein the VLP is flexuous.

5

6 10 A method as claimed in any preceding Claim  
7 wherein a cleavage site is incorporated on one of, or  
8 between, said first and second portions.

9

10 11 A method as claimed in any preceding claim,  
11 wherein a linker peptide is incorporated between the  
12 first and second portions.

13

14 12 A method as claimed in any preceding claim,  
15 wherein the second portion has a molecular weight of up  
16 to 10 kDa.

17

18 13 A method as claimed in any one of claims 1 to 11,  
19 wherein the second portion has a molecular weight of  
20 between 10 kDa and 30 kDa.

21

22 14 A method as claimed in any one of claims 1 to 11,  
23 wherein the second portion has a molecular weight over  
24 30kDa.

25

26 15 A method as claimed in any preceding claim,  
27 wherein the first portion is derived from a viral coat  
28 protein or a modified version thereof.

29

30 16 A method as claimed in any preceding claim,  
31 wherein the first portion is derived from a tobamovirus  
32 or a potexvirus.

33

34 17 A method as claimed in any preceding claim,  
35 wherein the second portion or the protein derived  
36 therefrom is a biologically or pharmaceutically active

1 or useful molecule.

2

3 18 A method as claimed in any one of claims 1 to 16,  
4 wherein the second portion or the protein derived  
5 therefrom is a diagnostic reagent.

6

7 19 A method as claimed in any one of claims 1 to 16,  
8 wherein the second portion or the protein derived  
9 therefrom is a food supplement.

10

11 20 A method as claimed in any preceding claim,  
12 wherein the virus particle is formed by a mixed  
13 assembly of chimaeric proteins together with some  
14 unmodified or less modified forms of the naturally  
15 occurring wild-type coat protein which forms the basis  
16 of the first portion.

17

18 21 A method as claimed in claim 20, wherein the  
19 chimaeric proteins and the unmodified or less modified  
20 forms of the naturally occurring wild-type protein are  
21 expressed from different sequences of nucleic acid.

22

23 22 A method as claimed in claim 21, wherein the  
24 different sequences are on the same piece of nucleic  
25 acid in the cell.

26

27 23 A method as claimed in claim 21, wherein the  
28 different sequences are on different pieces of nucleic  
29 acid in the cell.

30

31 24 A method as claimed in claim 20, wherein the  
32 chimaeric proteins and the unmodified or less modified  
33 forms of the naturally occurring wild-type protein are  
34 expressed from the same sequence of nucleic acid.

35

36 25 A method as claimed in claim 24, wherein the



1 chimaeric proteins and the unmodified or less modified  
2 forms of the naturally occurring wild-type protein are  
3 generated by co-translational modification, or are  
4 modified after translation.

5

6 26 A method as claimed in any preceding claim,  
7 wherein the virus from which the first portion is  
8 derived forms a particle with a relatively high pitch  
9 of helix.

10

11 27 A virus-like particle (VLP) comprising nucleic  
12 acid and protein, the protein having a first (viral)  
13 portion and a second (non-viral) portion.

14

15 28 A VLP as claimed in claim 27 wherein the nucleic  
16 acid comprises an origin of assembly sequence which  
17 permits initiation of assembly of the protein and  
18 nucleic acid into VLPs.

19

20 29 A VLP as claimed in either of claims 27 or 28,  
21 wherein the second portion (non-viral) of the protein  
22 is disposed on the outer surface of the VLP.

23

FIG. 1

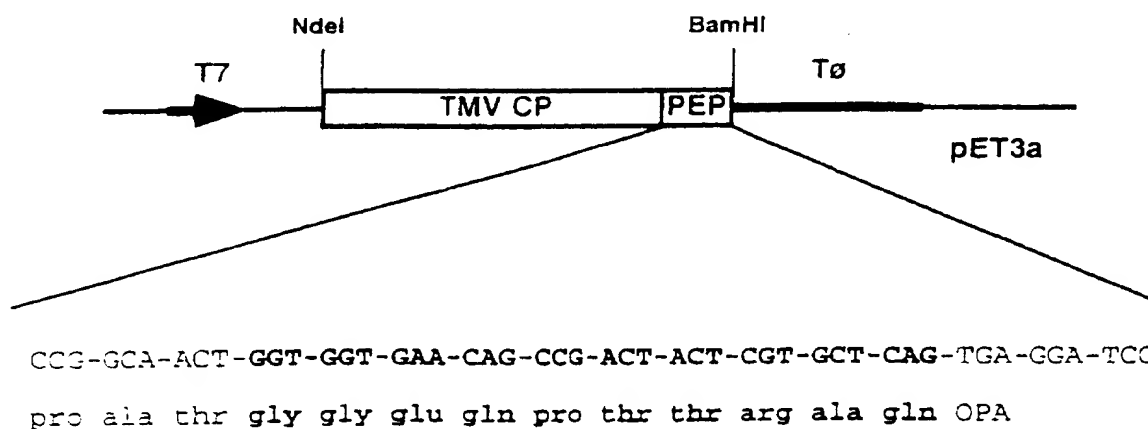


FIG. 2

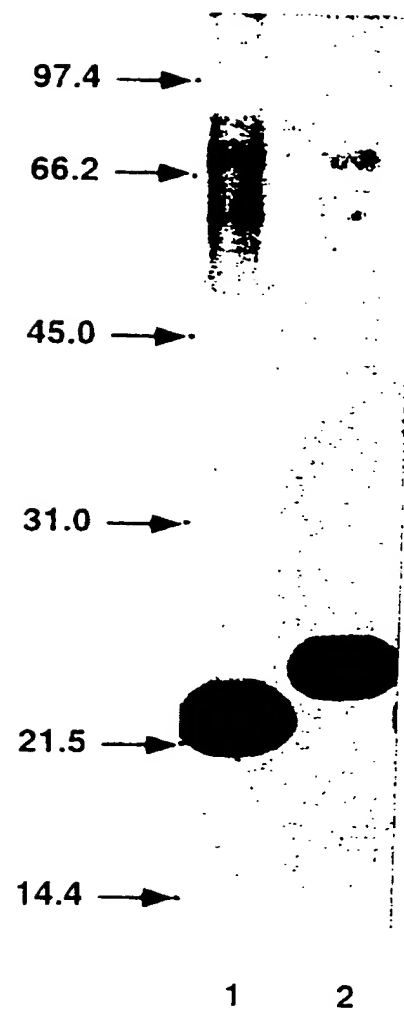
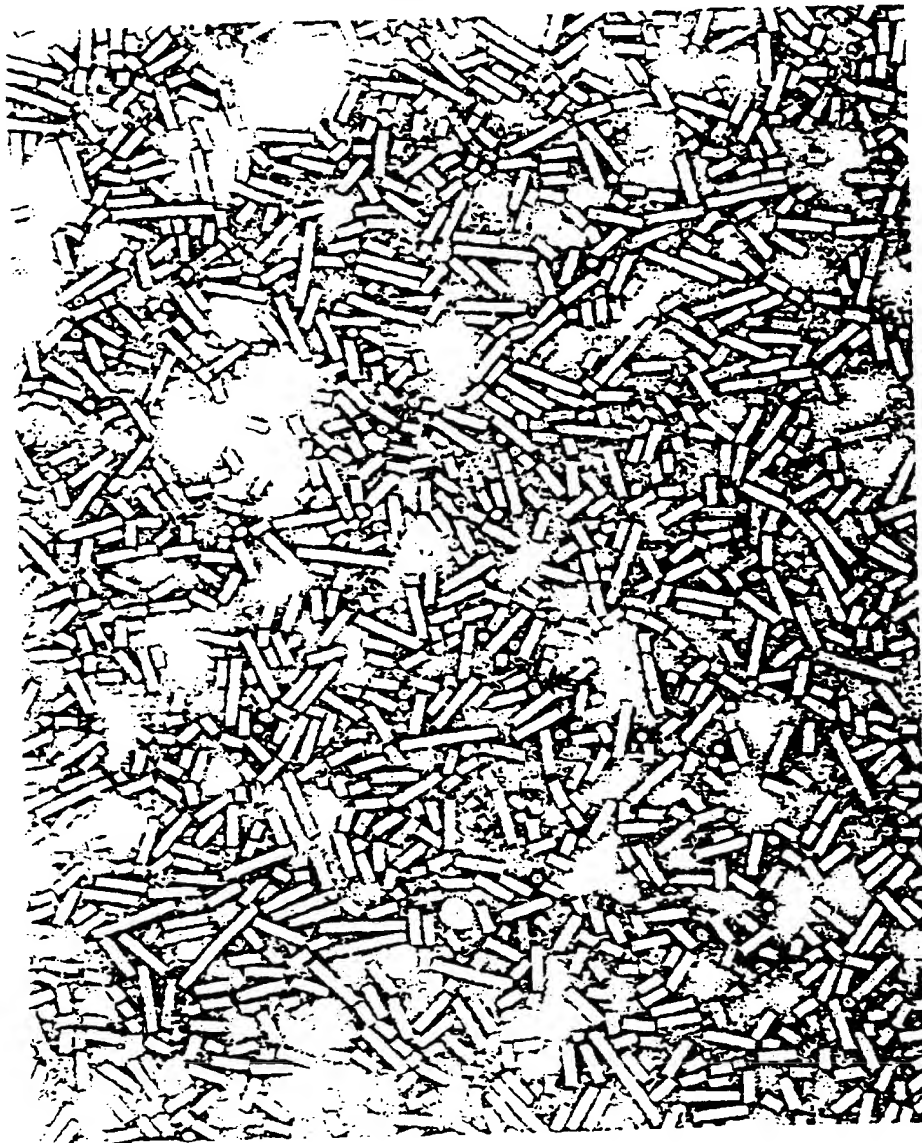


FIG. 3



pLiC.GFP-2A<sub>2</sub>311-CP

TCC GGA TCT AGA GCA CCT GTG AAA CAG CTG TTG AAT TTT GAC CTT AAG CTT CCG GGA GAC GTC GAG TCC AAC CCT GGG  
S G S S R A P V K Q L L L H F D L L K L A G D V E S H P G.

plilic.GFP-2A<sub>16H</sub>-CP

TCC GGA TCT ACG AAT TTT GAC CTT CTT AAG CTT GCG GGA GAC GTC GAG TCC AAC CTT GAG  
S G S R N F D L L K L A G D V E S H P G.

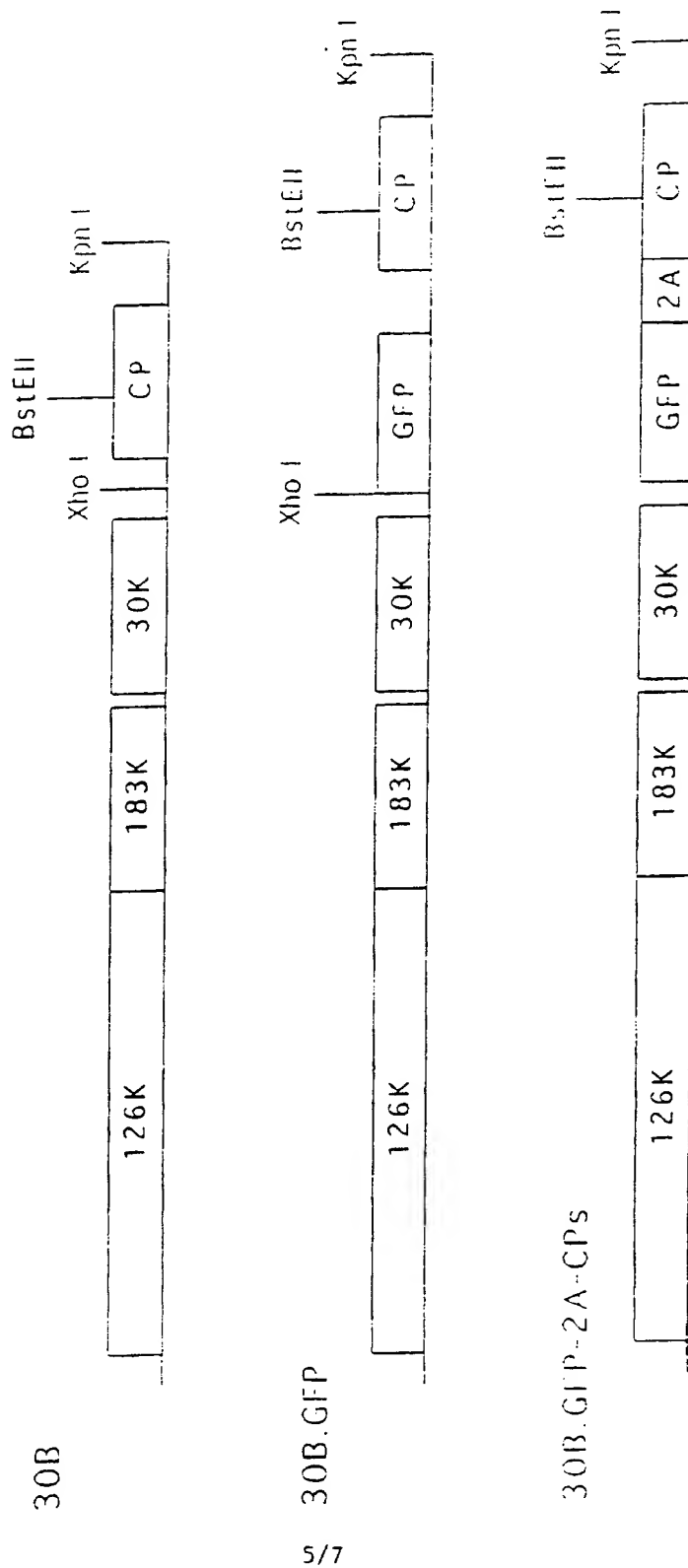
pl.it.GFP-2A<sub>1</sub>CR-CP

TCC GGA TCT AGA AAT TTT GAC CTT CTC AAG TTG GCG GGA GAC GTC GAG TCC ANC CCT GAG  
S G S R H F D L L K L A G D V E S H P G.

pl.it.GFP-2A<sub>90X</sub>-CP

TCC GGA "TCT" AGA GTC ACC GAG TTG CTT TAC CGG ATG AAG AGG GCC GAA ACA TAC TGT CCA AGG CCC TTG CTG GCA ATC CAC GCA ACT GAA GCG  
S G S R V T E L L Y R H K R A E T Y C P R P L L A I H P T E A.  
AGA CAC AAA CAG AAA ATT GTG GCA CCG GTG AAA CAG ACT TTG AAT TTT GAC CTT CTC ANG TTG GCG GGA GAC GTG GAG TGC AAC CCT GGG  
R H K Q K I V A P V K Q T L L H F D L L K L A G D V E S H P G.

Figure 5.



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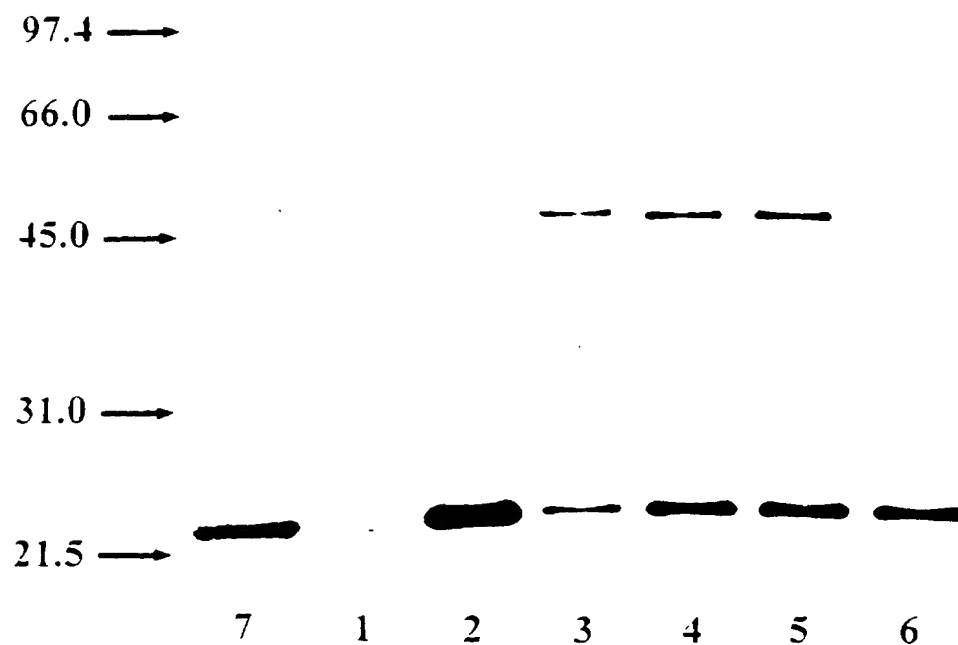
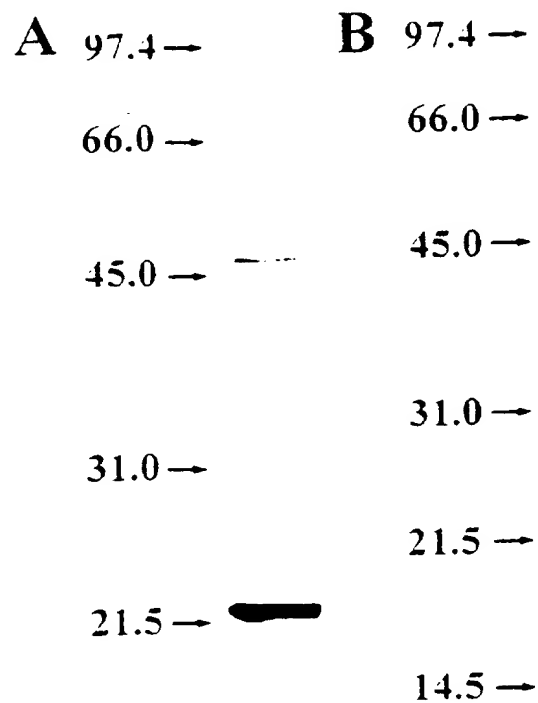
Figure 6.

Figure 7.



## INTERNATIONAL SEARCH REPORT

Internat. Application No  
PCT/GB 97/01065A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/87 C12N15/82 C12P21/02 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M.N. JAGADISH ET AL.: "High level production of hybrid Potyvirus-like particles carrying repetitive copies of foreign antigens in Escherichia coli" BIO/TECHNOLOGY., vol. 11, no. 10, October 1993, NEW YORK US, pages 1166-1170, XP002040652 see the whole document	1-6, 8-12, 15-18, 20-29
X	WO 96 05292 A (CONNAUGHT LABORATORIES LIMITED) 22 February 1996 see page 7, line 25 - page 12, line 27; figures 1-16	1-12, 15, 17, 20-29
X	WO 95 10624 A (BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 20 April 1995 see the whole document	1-12, 15, 17, 20-29

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

15 September 1997

Date of mailing of the international search report

23. 09. 97

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# INTERNATIONAL SEARCH REPORT

International patent family members

Inter Application No

PCT/JP 97/01065

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9605292 A	22-02-96	AU 3159995 A CA 2197446 A EP 0778888 A	07-03-96 22-02-96 18-06-97
WO 9510624 A	20-04-95	DE 4335025 A AU 7812094 A EP 0724643 A JP 9503665 T	20-04-95 04-05-95 07-08-96 15-04-97

Form PCT/ISA/210 (patent family annex) (July 1992)